The Journal of Infectious Diseases

MAJOR ARTICLE

Monitoring HLA-A2-Restricted T-Cell Responses and BCLA-Specific Serostatus During Human Latent *Toxoplasma gondii* Infection Suggests the Implication of CD8+ T Cells in Parasite Containment

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Background. T cells control *Toxoplasma gondii* (*T. gondii*) parasite infection but, our understanding of parasite –specific CD8+ T-cell responses in humans remains scarce. Here, we studied 56 HLA-A2+ healthy blood donors, including 40 infected subjects.

Methods. Beyond routine *T. gondii* serodiagnosis, we quantified IgG specific for the bradyzoite-restricted Brain Cyst Load-associated Antigen (BCLA) and we enumerated blood-circulating CD8+ T cells specific for 29 *T. gondii* antigenic peptides.

Results. We identified a set of 16 epitopes that stimulates detectable CD8+ T-cell responses in 50% routine *T. gondii*–seropositive subjects, yet none was a universal immunodominant epitope. Among individuals with either positive BCLA-specific serology and/or detectable *T. gondii*–specific T-cell response, *T. gondii*–specific ELISPOT responses were inversely correlated with BCLA-specific IgG titers, suggesting that stronger CD8+ T-cell responses may contribute to reduce chronic parasite burden or that antibody levels and CD8+ T cell memory differentially persist. Furthermore, 2 out of 56 subjects displayed null *T. gondii*–specific humoral responses across 4 serological assays, but had measurable CD8+ T-cell responses to 2 *T. gondii* peptides.

Conclusions. This study sheds light on *T. gondii*–specific immune responses in humans and rationalizes the toolkits for immune monitoring of human latent *T. gondii* infection.

Keywords. *Toxoplasma gondii*; humans; CD8+ T cell; serodiagnostic; latent infection.

Toxoplasma gondii (*T. gondii*) is an obligate intracellular opportunistic parasite that belongs to the phylum Apicomplexa. Humans typically become infected by ingesting cysts in undercooked meat or oocysts shed from cats in the environment, making *T. gondii* the 3rd costliest foodborne pathogen in the United States [1]. Following ingestion, *T. gondii* converts into fast-replicating tachyzoites which spread systemically during the acute phase of infection. Tachyzoites respond to extrinsic stressors [2, 3] and convert into bradyzoites, which are encysted latent stages chronically persisting within muscular, retinal, and neuronal cells [4].

In humans, it is well accepted that primary *T. gondii* infection elicits parasite-specific humoral responses. To detect anti–*T. gondii* immunoglobulins, commercially available serodiagnostic tests use tachyzoite lysates (Biorad Platelia®) or antigens like SAG1 and GRA8 (Abbott Alinity®) that are expressed at higher levels in tachyzoites than in bradyzoites (see ToxoDB.org database [5]). Furthermore, some *T. gondii*–exposed individuals display humoral responses to bradyzoite or cyst wall antigens [6–8]. In agreement, around 60% individuals tested positive by routine serodiagnosis concomitantly display seroreactivity against a cyst wall antigen called Brain Cyst Load-associated Antigen (BCLA/MAG2) [9]. Humoral responses specific for the bradyzoite serological marker (BSM) was also associated with chronic toxoplasmosis in mouse infection models, and seroreactivity to BSM is observed in humans [10].

In mouse models, CD8+ T cells play a critical role in *T. gondii* protective immunity. T cells and T cell–produced IFN-γ [11, 12] seem essential both during acute and chronic infection for dampening cyst load in the brain [13–16]. In humans, the major role of T cells during *T. gondii* infection is illustrated by the close association between depressed cellular

Received 09 November 2025; accepted 18 June 2026; published online 1 July 2026

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The Journal of Infectious Diseases®

https://doi.org/10.1093/infdis/jiag333

immunity occurring upon HIV/AIDS and cerebral toxoplasmosis [17].

Measurements of *T. gondii*–specific T-cell responses in humans were initiated in the mid-twentieth century, when clinicians assessed dermal sensitivity to *T. gondii* tachyzoite lysates as a tool to screen exposed individuals [18, 19]. Recently, a handful of studies identified *T. gondii* epitopes presented by HLA class I molecules, particularly the HLA-A2 supertype molecules [20–24]. In some instances, the presence of effector memory CD8+ T cells specific for these HLA-A2-presented epitopes was tested in *ex vivo* IFN-γ enzyme-linked immunospot (ELISPOT) assays on peripheral blood mononuclear cells (PBMC), showing responses ranging from approximately 100 to approximately 3000 IFN-γ Spot Forming Cells (SFC)/10^6 PBMC in all *T. gondii*–seropositive individuals (up to 4 individuals tested in each study) versus no response in *T. gondii*–seronegative individuals [20–22]. The small number of subjects tested for each category (seropositive vs seronegative) and the heterogeneity of the serological techniques used to define *T. gondii* serodiagnosis limit the extrapolation of these results to a broader adult population.

In the present study, we assessed the presence of *T. gondii*–specific effector memory CD8+ T cells specific for a panel of 29 peptides derived from antigens expressed by tachyzoites or bradyzoites [20–22, 24], with the exception of the FLFAWITYV epitope derived from a zinc finger protein and described in 2024 [23], in PBMC from 56 HLA-A2+ healthy adult blood donors.

METHODS

Study Population

The study was carried out on plasma and PBMC from blood donors (N = 282, including 56 HLA-A2+ subjects analyzed by ELISPOT) recruited between June 2020 and September 2022 in Toulouse, France. Blood donor characteristics, plasma and PBMC cryopreservation, HCMV and *T. gondii* serodiagnosis, and HLA-A2 expression are detailed in the Supplementary Methods.

Antigenic Peptides

The 29 *T. gondii* epitopes tested in this study were all previously reported as potential HLA-A*02:01 ligands. They are listed along with their main characteristics and study of reference in Table 2. Cellular Technology Limited (CEF) is a pool of 23 pan-HLA-presented peptide epitopes from HCMV, Epstein–Barr virus (EBV), and influenza virus that have been selected for their efficacy to recall CD8+ T cells [25].

Quantification of Enzyme-Linked Immunospot Responses

Enzyme-linked immunospot analyses were performed as detailed in the Supplementary Methods. To harmonize the ELISPOT approach, we followed suggestions by the Global CRO Council in Bioanalysis [26]. We defined a technical limit of detection (LOD) in order to distinguish the positive responses from the background-related nonspecific signal detected in mock-stimulated PBMC (ie, with DMSO-containing buffer, without peptide), as illustrated in Supplementary Figure 1*A*. *T. gondii* peptides #1 to #29 were tested separately on PBMC from each subject. An ELISPOT response was designated as not detectable if the number of SFC obtained with peptide activation (SFC peptide) was below the LOD. Next, if the SFC peptide value was above the LOD, the subject's background response (ie, SFC with DMSO-containing buffer) was subtracted from the SFC value obtained with each peptide (SFC peptide minus SFC buffer) to infer the background-corrected peptide-specific response (as illustrated in Supplementary Figure 1*B*). A subject was then considered as ELISPOT responder if she/he had a positive background-corrected ELISPOT response to at least 1 peptide among the 29 *T. gondii* peptides, or designated as non-responder otherwise. In some instances, we summed IFN-γ SFC/10^6 PBMC values obtained with all 29 *T. gondii* peptides in order to evaluate the overall magnitude of the subject's T-cell response. Supplementary Figure 2 shows representative examples of ELISPOT responses measured with *T. gondii* peptide #11 in PBMC from 7 different routine *T. gondii*–seropositive subjects.

Statistical Analyses

See Supplementary Methods.

RESULTS

Characteristics of the Enzyme-Linked Immunospot–Tested HLA-A2+ Healthy Adult Subjects Assessed in This Study

We randomly selected 16 routine HLA-A2+ *T. gondii*–seronegative (anti-SAG1 IgM-negative and anti-SAG1/GRA8 IgG-negative) and 40 routine HLA-A2+ *T. gondii*–seropositive (anti-SAG1 IgM-negative and anti-SAG1/GRA8 IgG-positive) individuals (Figure 1*A*) from a primary biobank of plasma and PBMC from healthy adult blood donors (N = 282) aged 18–70 years. Blood donors in the primary biobank comprised 49% women and had a median age of 42.5 (IQR 27) years (Table 1). Thirty-seven (37) percent and 53% of these individuals were positive for anti-HCMV IgG and HLA-A2 expression (Table 1), consistent with HCMV seropositivity [27] and HLA A2 frequency in France (http://www.allelefrequencies.net/). In this primary biobank, 46% of subjects were routine *T. gondii* seronegative and 53% were routine *T. gondii* seropositive. With regard to age, 68% of subjects aged 18 to 42 years versus 23% of subjects aged 43 to 70 years were routine *T. gondii* seronegative, respectively (Figure 1*B*). This age-dependent increase in *T. gondii*–specific seroprevalence is in agreement with previous reports from various countries [28–30].

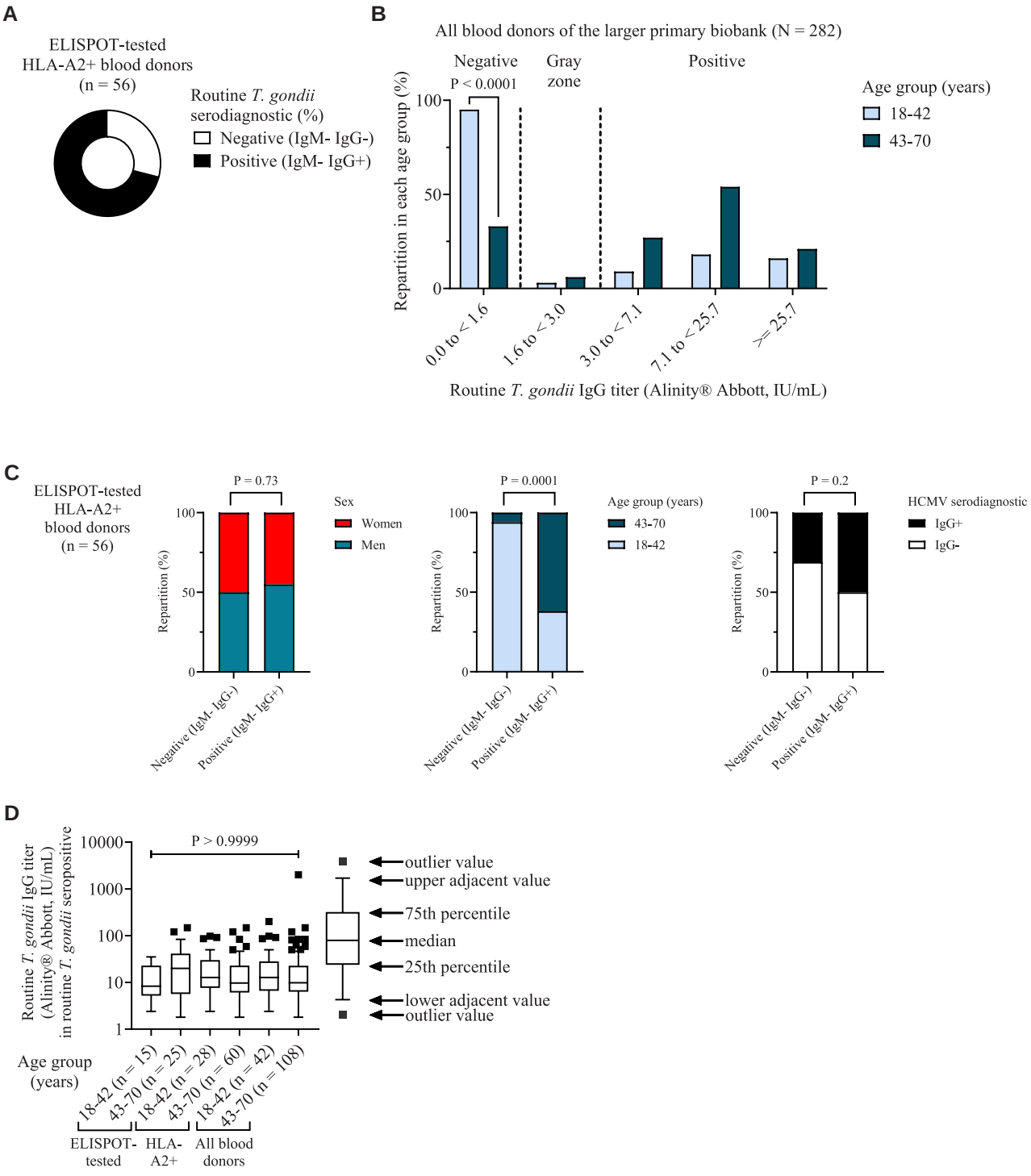

Figure 1. Routine *T. gondii*–seropositive HLA-A2+ healthy adult subjects assessed in ELISPOT analyses display characteristics of French blood donors with past *T. gondii* infection. *A*, Anti-SAG1 or GRA8 IgG titers in the primary blood donor bank. ELISPOT-tested subjects represent a sub-group of a larger biobank of plasma and PBMC, collected from blood donors (N = 282) aged 18 to 70 y who donated their blood between June 2020 and September 2022 in Occitania region (South-West of France), and for whom anti-SAG1 or GRA8 IgG titers were quantified with the Alinity® test. Numbers of routine *T. gondii*–seronegative individuals were compared between the 2 age groups with *P* value (χ^2 test) displayed on the figure. *B*, Routine *T. gondii* serodiagnosis in ELISPOT-tested HLA-A2+ subjects (n = 56). Sixteen subjects were anti-SAG1 IgM negative and anti-SAG1 and GRA8 IgG negative (designated as "routine *T. gondii*–seronegative" subjects), and 40 subjects were anti-SAG1 IgM negative and anti-SAG1 or GRA8 IgG positive (designated as "routine *T. gondii*–seropositive" subjects). *C*, Distribution of sex, age, and HCMV serostatus among the HLA-A2+ routine *T. gondii*–seropositive subjects tested in ELISPOT (n = 40) and the HLA-A2+ routine *T. gondii*–seronegative subjects tested in ELISPOT (n = 16). Proportions displayed were compared with χ^2 tests and *P* values are displayed on the figure. *D*, Anti-SAG1 or GRA8 IgG titers in routine *T. gondii*–seropositive (IgM− IgG+) blood donors aged 18–42 y and 43–70 y from the primary blood bank, HLA-A2+ blood donors, and ELISPOT-tested HLA-A2+ blood donors. Values were compared with a Kruskal–Wallis test and Dunn's correction for multiple comparisons. The figure shows medians, IQR, and *P* values for all 2 by 2 comparisons.

Table 1. Summary Characteristics of the Studied Blood Donors Recruited Through the French Transfusion Public Service (EFS) in the South-West of France (Administrative Region of Occitania)

	All Blood Donors (Larger Primary Blood Bank)	All HLA-A2+ Blood Donors	ELISPOT-Tested HLA-A2+ Blood Donors
N	282	149	56
Women (%)	49	45 ($P = .4$)	46 ($P = .8$)
Age median IQR (year)	42.5 IQR 27	41 IQR 27 ($P = .8$)	40.5 IQR 27 ($P = .7$)
HCMV seroprevalence (%)	37	39 ($P = .6$)	45 ($P = .4$)
HLA-A2+ (%)	53	100 ($P < .0001$)	100
Routine *T. gondii* IgM− IgG− (%)	46	40 ($P = .3$)	29 ($P = .12$)
Routine *T. gondii* IgM+ IgG− (%)	0	0	0
Routine *T. gondii* IgM+ IgG+ (%)	1	1 ($P = .7$)	0 ($P = .5$)
Routine *T. gondii* IgM− IgG+ (%)	53	59 ($P = .2$)	71 ($P = .103$)
Age of routine *T. gondii* IgM− IgG− median IQR (year)	31 IQR 20	30.5 IQR 15 ($P = .9$)	29 IQR 23 ($P = .3$)
Age of routine *T. gondii* IgM− IgG+ median IQR (year)	52.5 IQR 24	51.5 IQR 21 ($P = .6$)	49.5 IQR 15 ($P = .5$)

P values in the "All HLA-A2+ Blood Donors" column indicate the comparison with the larger primary blood bank consisting of all blood donors, while *P* values in the "ELISPOT-Tested HLA-A2+ Blood Donors" column indicate the comparison with all HLA-A2+ blood donors. Statistical significance was computed using the Mann–Whitney test for continuous variables or the χ^2 test for categorical variables.

Enzyme-linked immunospot–tested HLA-A2+ subjects displayed no difference in gender, age, HCMV seroprevalence, and *T. gondii* seroprevalence compared with the HLA-A2+ blood donors of the primary biobank ($P > .103$; Table 1). In addition, the 16 routine *T. gondii*–seronegative subjects tested in ELISPOT were not different from the 40 routine *T. gondii*–seropositive subjects in terms of sex ratio and HCMV serostatus, but, as expected, they were younger ($P < .0001$; Figure 1*C*). Of note, although *T. gondii* seroprevalence is higher in adults over 43 years old, the titers of *T. gondii*–specific IgG in routine *T. gondii*–seropositive subjects were comparable across the 3 groups (primary biobank, HLA-A2+ subjects, and ELISPOT-tested HLA-A2+ subjects), regardless of age ($P > .9$; Figure 1*D*). In conclusion, we have characterized a subset of 56 HLA-A2+ healthy adult subjects who display similar features as those of the HLA-A2+ subjects they are sampled from.

T. gondii–specific Effector CD8+ T-Cell Responses in Routine *T. gondii*–Seropositive HLA-A2+ Subjects

Twenty-nine *T. gondii* peptides (8–11 amino acid long) published to be actual or putative ligands of HLA-A2 [20–22, 24] were used in this work (Table 2). They originate from diverse proteins expressed by tachyzoite and/or bradyzoite stages from type II *T. gondii*, a clonal lineage likely to be present in France. Remarkably, out of the 29 *T. gondii* peptides, 13 peptides (#1, #3, #4, #8, #9, #10, #18, #19, #20, #21, #23, #25, and #29) yielded background-corrected peptide-specific IFN-γ SFC values either undetectable in all subjects or in the first quartile of positive responses (ie, equal to or below 38 IFN-γ SFC/10^6 PBMC) in up to 3 subjects among the 40 routine *T. gondii*–seropositive individuals (Figure 2*A*). The remaining 16 peptides (#2, #5, #6, #7, #11, #12, #13, #14, #15, #16, #17, #22, #24, #26, #27, #28), designated from here as "16-peptide set," induced background-corrected ELISPOT responses above the first quartile of positive responses (ie, above 38 IFN-γ SFC/10^6 PBMC) in at least one individual. Both the proportion of ELISPOT responders and the response intensity, measured as the summed ELISPOT responses to peptides, were identical when considering all 29 *T. gondii* peptides and the 16-peptide set and higher than observed for the remaining 13-peptide set ($P < .01$; Supplementary Figure 1*C*). Peptides #11, #14, and #17 induced ELISPOT responses in the largest number of subjects (ie, 5–7 subjects) (Figure 2*A*). These 3 peptides appear as the most shared immunogenic peptides although none was a "universal" HLA-A2-restricted *T. gondii* epitope. Noteworthy, out of the 40 routine *T. gondii*–seropositive HLA-A2+ individuals, 20 subjects (50%) responded to the 16-peptide set and all displayed responses to 1, 2, or 3 *T. gondii* peptides (Figure 2*B*).

Of note, the overall magnitude of the *T. gondii*–specific CD8+ T-cell responses in the 20 individuals responsive to 16-peptide set in ELISPOT was 5.3-fold lower than the ELISPOT response measured upon stimulation with the pool of CEF peptides ($P = .0003$; Figure 2*C*). It was observed that 4 μg/mL peptide concentration elicited the maximum response, indicating that lower responses from *T. gondii* peptide compared to CEF peptides were not caused by underdosing (Figure 2*D*).

We observed no difference in the proportion of individuals who were ELISPOT responders to *T. gondii* according to HCMV serodiagnosis ($P > .9$; Supplementary Figure 3*A*). However, we noticed that adults older than 43 years displayed a higher proportion of ELISPOT responders to the 16-peptide set than younger adults (64% vs 27%, $P = .02$; Supplementary Figure 3*A*). Also, more women were ELISPOT responders to the 16-peptide set than men (64% vs 36%), although this difference did not reach significance ($P = .06$; Supplementary Figure 3*A*). Nevertheless, the overall magnitude of the *T. gondii*–specific CD8+ T-cell responses, as measured by summed IFN-γ SFC for the 16-peptide set, was similar regardless of age and sex among ELISPOT responders ($P > .9$;

Table 2. Description of the 29 HLA-A*02:01-Binding Epitope Peptides Used in ELISPOT Assays

Peptide #	Epitope Sequence	Source Protein	ToxoDB ID	Stage Expression	HLA-A2 Binding Percentile Rank	Reference Study
1	VVFVVFMGV	GRA6	TGME49_275440	T = B	2	[20, 21]
2	FMGVLVNSL	GRA6	TGME49_275440	T = B	0.38	[20, 21]
3	FLVPFVVFL	GRA3	TGME49_227280	T = B	0.03	[20, 21]
4	FLSLSLLVI	SRS49D	TGME49_207160	B > T	0.84	[21]
5	FMIAFISCFA	SRS49C	TGME49_207150	B > T	2.4	[21]
5	FMIAFISCFA	SRS49D	TGME49_207160	B > T	2.4	[21]
6	FVIFACNFV	SRS49B	TGME49_207140	B > T	1.9	[21]
7	FMIVSISLV	SRS49B	TGME49_207140	B > T	0.2	[21]
8	FLLGLLVHV	SRS57	TGME49_308020	T > B	0.02	[21]
9	FLTDYIPGA	SRS57	TGME49_308020	T > B	0.03	[21]
10	ITMGSLFFV	SRS52A	TGME49_315320	T = B	0.19	[21]
11	GLAAAVVAV	GRA5	TGME49_286450	T = B	0.05	[21]
12	VLLPVLFGV	MIC1	TGME49_291890	T > B	0.03	[21]
13	FAAAFFPAV	M2AP	TGME49_214940	T = B	0.32	[21]
14	FLADLLHSV	TER1P	TGME49_206350	T > B	0.01	[22]
15	FLDRALLTL	GOT1	TGME49_295460	T = B	0.02	[22]
16	ALLALLIYA	LMBR1	TGME49_222200	T = B	0.32	[22]
17	FVGSYLVIV	PLA2	TGGT1_273620	Not available	0.34	[22]
18	ALAALAPSLEV	PLA	TGME49_306330	T = B	0.36	[24]
19	ALDINLPNV	RPS17	TGME49_207840	T = B	0.03	[24]
20	FLAQVIVL	EF-1-ALPHA	TGME49_286420	T = B	0.54	[24]
21	FVLELEPEWTV	DSK2	TGME49_223680	T = B	0.27	[24]
22	GLAETEVPV	GRA2	TGME49_227620	T = B	0.06	[24]
23	HLMDEAVEV	Transaldolase	TGME49_229360	T = B	0.01	[24]
24	ILDEATSAL	ABCB1	TGME49_260310	T = B	0.02	[24]
25	LLSDLSVRL	GRA29	TGME49_269690	T = B	0.02	[24]
26	MLYAGAPTTV	IST	TGME49_240060	T > B	0.12	[24]
27	VMSELEFYL	GRA23	TGME49_297880	T = B	0.03	[24]
28	YLFKEGVIVV	RPS10	TGME49_275810	T = B	0.04	[24]
29	YLSPIASPLL	60S ribosomal protein L7a, putative	TGME49_313560	T = B	0.1	[24]

Twenty-nine *T. gondii*–derived peptides putatively binding HLA-A*02:01 were selected from 4 previous studies [20–22, 24]. We matched peptides to *T. gondii* proteins in ME49 (type II) reference strain, with the exception of peptide #17 that has ToxoDB accession number TGGT1_273620, and we used ToxoDB (https://toxodb.org/toxo/app) to report the respective expression of mRNA in *T. gondii* tachyzoites (T) versus bradyzoites (B). mRNA similarly expressed at both stages was designated as "T = B". Alternatively, > and < symbols were used to indicate more than 4-fold increase or decrease in mRNA expression between the 2 differentiation stages. Servers from the Immune Epitope Database (IEDB, https://nextgen-tools.iedb.org/pipeline) were used to predict the binding of each peptide to the HLA-A*02:01 allele using the NetMHCpan 4.1 prediction model, based on [31]. NetMHCpan 4.1 determines the HLA-A2 binding percentile rank with a computational approach by comparing the predicted affinity of each peptide to HLA-A2 against those of a set of random peptides from SWISSPROT database. A lower HLA-A2 binding percentile rank corresponds to a higher binding affinity.

Supplementary Figure 3*B*), suggesting that while the proportion of responders may be influenced by age and sex, once induced, the intensity of the response remains the same.

Together, these data indicate that (i) none of the 29 peptides tested here is a universal HLA-A2 epitope able to restimulate CD8+ T cells from a majority of *T. gondii*–exposed HLA-A2+ individuals, (ii) *T. gondii*–specific peripheral CD8+ T-cell responses assessed with this panel of 29 epitopes are of low magnitude, compared to CEF-specific CD8+ T-cell responses, and (iii) half of the 40 routine *T. gondii*–seropositive HLA-A2+ individuals display ELISPOT responses to at least one peptide from the set of 16 *T. gondii* peptides.

T. gondii–specific Effector CD8+ T-Cell Responses in Routine *T. gondii*–Seronegative and BCLA-specific IgG-Negative HLA-A2+ Subjects

To estimate the relevance and specificity of the 16-peptide set, we sought to test its ability to restimulate PBMC from subjects who displayed negative *T. gondii*–specific serology. Since the assay we used in the present study to determine routine *T. gondii* serodiagnosis is biased for the detection of antibodies specific for tachyzoite-expressed SAG1 or GRA8, we also tested the seroreactivity to the bradyzoite-restricted BCLA protein [9]. Five (5) out of 16 (31%) routine *T. gondii*–seronegative HLA-A2+ subjects actually carried anti-BCLA IgG, suggesting the presence of a *T. gondii* chronic infection along with a routine antibody response below threshold. Strikingly and as expected, the proportion of ELISPOT responders to the 16-peptide set was higher in subjects who were routine *T. gondii*–seropositive or BCLA-specific IgG positive, or both (51%) compared to subjects who were negative for both IgG (18%, $P = .0489$, Figure 3*A*). Intriguingly, 2 out of 11 subjects who were both routine *T. gondii*–seronegative and BCLA-specific IgG negative responded in ELISPOT assay to 2 peptides among peptides #14,

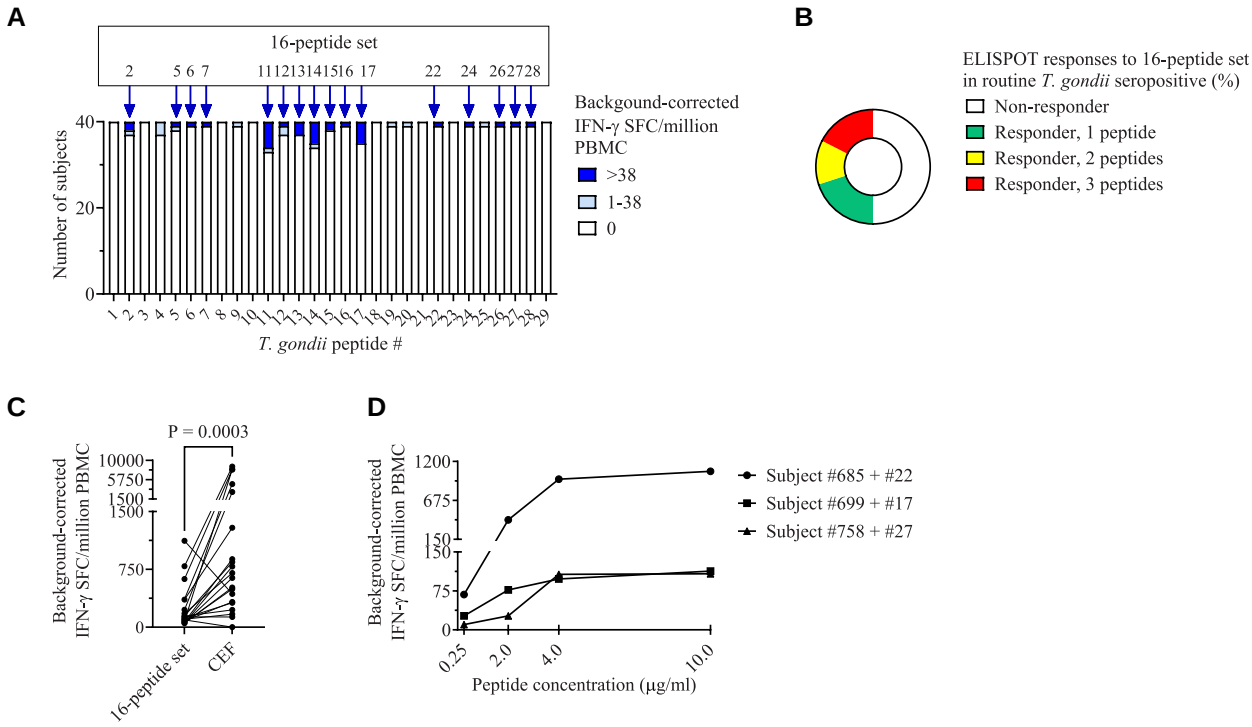

Figure 2. Blood donors with past *T. gondii* infection display low magnitude CD8+ T-cell ELISPOT responses to HLA-A2-presented *T. gondii* epitope peptides. *A*, *T. gondii* peptide ELISPOT responsiveness in routine *T. gondii*–seropositive subjects (n = 40), whose PBMC were tested in ELISPOT assays with *T. gondii* peptide #1 to #29 individually. Each bar shows, for each peptide, the number of subjects responding to the peptide with the indicated magnitude (expressed as IFN-γ SFC/10^6 PBMC). Peptides indicated by arrows above the bar constitute the 16-peptide set (#2, #5, #6, #7, #11, #12, #13, #14, #15, #16, #17, #22, #24, #26, #27, and #28), which elicit ELISPOT responses above the first quartile of positive responses (above 38 IFN-γ SFC/million PBMC) in at least one subject. *B*, CD8+ T-cell responsiveness to the 16-peptide set in routine *T. gondii*–seropositive subjects (n = 40). Subjects were classified as non-responders (all responses equal to 0 IFN-γ SFC/10^6 PBMC) or responders to the indicated number of peptides. *C*, Comparison of summed ELISPOT responses to the 16-peptide set and ELISPOT response to CEF peptide pool among ELISPOT responders (n = 20) to the 16-peptide set of *T. gondii*. *D*, Avidity of CD8+ T cells specific for *T. gondii* peptides in 3 routine *T. gondii*–seropositive subjects (subject #685, #699, and #758), whose PBMC were activated with increasing concentrations of peptide #22, #17, or #27, as indicated on the figure, or DMSO, as a control, before enumeration of IFN-γ SFC in ELISPOT assays. Values in (*C*) were compared with a Mann–Whitney test or a Wilcoxon matched-pairs signed rank test, and the figure shows medians, IQR, and *P* values.

#16, and/or #17 (Figure 3*B*). Further analyses of these 2 subjects revealed that both had no seroreactivity to a whole *T. gondii* lysate, as determined by the LDBio-Toxo II IgG immunoblot assay. In addition, none of these 2 subjects displayed IgG specific for the other bradyzoite-specific marker BSM. We suspect that T-cell responses may result from exposure to other human or animal pathogens, as several of them express proteins containing sequences that are either identical to the epitope peptides tested here, or with single conservative substitutions that maintain HLA-A2 binding (Supplementary Table 1).

In conclusion, we found a significant association between *T. gondii*–positive serodiagnosis (based on both tachyzoite and bradyzoite-specific assays) and ELISPOT responsiveness to the 16-peptide set. Nevertheless, 3 of these peptides (#14, #16, and #17) could restimulate T cells from 2 subjects devoid of tachyzoite and BSM- and BCLA-specific antibodies, suggesting an uncoupling of humoral and cellular responses to *T. gondii* in a subgroup of infected persons or unspecific reactions to homologous HLA-A2 peptides from other pathogens.

Can CD8+ T-Cell Enzyme-Linked Immunospot Response Be Used to Evaluate T Cell–Mediated Containment of Latent *T. gondii* Infection?

In mice, BCLA-specific IgG titers were found to mirror brain parasite load but in humans, a fraction of routine *T. gondii*–seropositive individuals are devoid of BCLA-specific IgG [9]. A possible explanation of this finding is that some people may clear *T. gondii* throughout latent infection, resulting in low levels of BCLA-specific IgG. Since CD8+ T cells play a prominent role in the control of acute and chronic stages of *T. gondii* infection in mouse models, we hypothesized that CD8+ T cells may be involved in parasite containment and/or clearance in humans as well. If this hypothesis is true, we predict that higher CD8+ T-cell responses should be associated with increased likelihood of negative anti-BCLA IgG serology in individuals who display an ELISPOT response and/or a BCLA-specific serology, as a result of decreased latent parasite burden mediated by T cells.

To test this prediction, we selected all subjects that displayed a positive BCLA-specific IgG serology or ELISPOT response to the 16-peptide set, or both. Thirty-three (33) out of

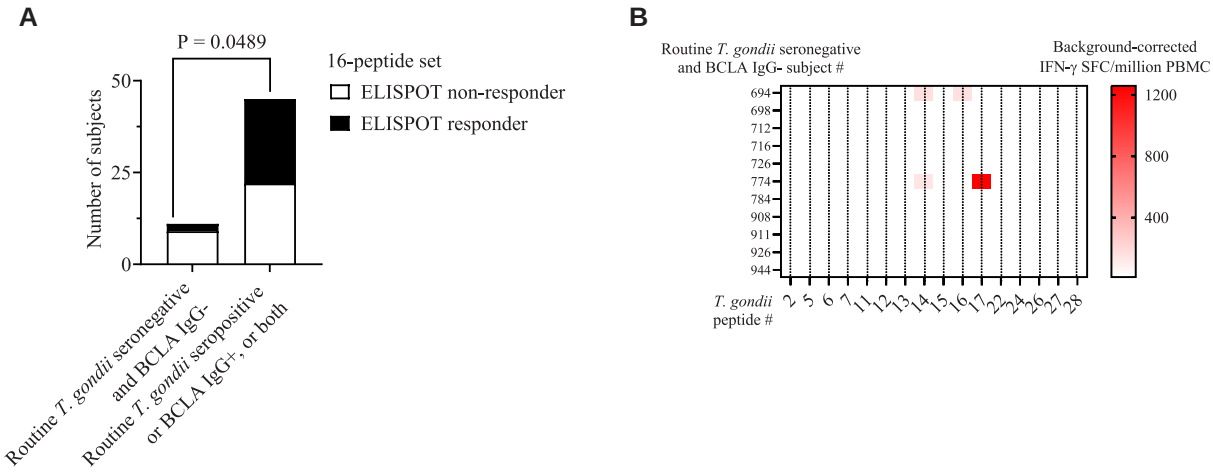

Figure 3. ELISPOT responses to the 16-peptide set in subjects who are both routine *T. gondii* seronegative and anti-BCLA IgG negative. *A*, Number of subjects who were *T. gondii* ELISPOT non-responders versus responders to the 16-peptide set according to routine *T. gondii* and anti-BCLA serostatus. Proportion was compared with a χ^2 test and the *P* value is displayed on the figure. *B*, Ranges of IFN-γ SFC/10^6 PBMC obtained with each *T. gondii* peptide from the 16-peptide set, for each of the 11 routine *T. gondii*–seronegative subject who was also anti-BCLA IgG negative.

the 56 HLA-A2+ blood donors responded to these inclusion criteria ("group B") (Figure 4*A*). Among these, there was a moderate decreasing monotonic relationship between BCLA-specific IgG titers and the overall ELISPOT responsiveness to *T. gondii* peptides, represented as the sum of ELISPOT responses to the 16-peptide set (Spearman's rank correlation coefficient $r = -0.5$, $P = .007$; Figure 4*B*). In line with this observation, among group B subjects, ELISPOT responses of individuals devoid of anti-BCLA IgG (226 (IQR 522) IFN-γ SFC/million PBMC) were 3.9-fold higher than ELISPOT responses of individuals displaying anti-BCLA IgG (58 [IQR 105] IFN-γ SFC/million PBMC, $P = .0017$; Figure 4*C*). As a control, no difference between CEF-specific ELISPOT responses was observed between these 2 groups of individuals ($P > .7$; Figure 4*D*). We further attempted unsupervised analyses of relationships between the variables investigated here, using Factor Analysis of Mixed Data. We observed that most (up to 20%) inter-individual variability was driven by BCLA IgG titers (continuous variable), BCLA_serology (categorical variable), and ELISPOT responses to *T. gondii* peptides #2, #11, and #13 (Supplementary Figure 4*A*). However, there was no obvious correlation between the cluster of peptides #2, #11, and #13 and BCLA-specific IgG titers (Supplementary Figure 4*B*).

In conclusion, among subjects who are likely chronically infected with *T. gondii* and characterized by a positive BCLA serology or a detectable CD8+ T-cell response, or both, there is a negative correlation between the magnitude of CD8+ T-cell ELISPOT responses to the 16-peptide set and the BCLA-specific serostatus, suggesting that strong CD8+ T-cell responses may contribute to curtail the infection in humans. Alternatively, these observations may reflect unphased kinetics in parasite-specific antibody and cellular responses.

DISCUSSION

By investigating the relevance of 29 *T. gondii* HLA-A2 ligands in 56 human individuals evaluated with routine *T. gondii* serodiagnosis and a recently described bradyzoite-specific serological assay [9], our work sheds light on peripheral blood parasite-specific CD8+ T-cell responses during latent human *T. gondii* infection. The main messages conveyed by our study are that effector memory CD8+ T-cell responses to these 29 *T. gondii* epitope peptides are generally weak, without evidence of a universal epitope, increase with age, and may be uncoupled from humoral responses. Yet, our data suggest that the few individuals able to mount the most robust CD8+ T-cell responses to *T. gondii* may be advantaged to more effectively control latent infection, in line with the hypothesis that a fraction of individuals may clear the infection [32].

None of the 29 *T. gondii* peptides was recognized by all or most routine *T. gondii*–seropositive HLA-A2+ subjects, although they originate from antigenic proteins expressed in the type II clonal lineage that is predominant in Europe [33]. The absence of a universal *T. gondii* epitope contrasts with other common chronic infections, such as those induced by HCMV or EBV, where immunodominant HLA-A2-presented epitopes have been identified [34, 35]. It is possible that immunodominant T-cell antigens have not yet been discovered, due to their inefficient processing and presentation in the human monocytes used to identify HLA-A2 ligands [24], or due to their presence in source antigens that were not screened for putative epitopes [20–22], or due to poor prediction by HLA

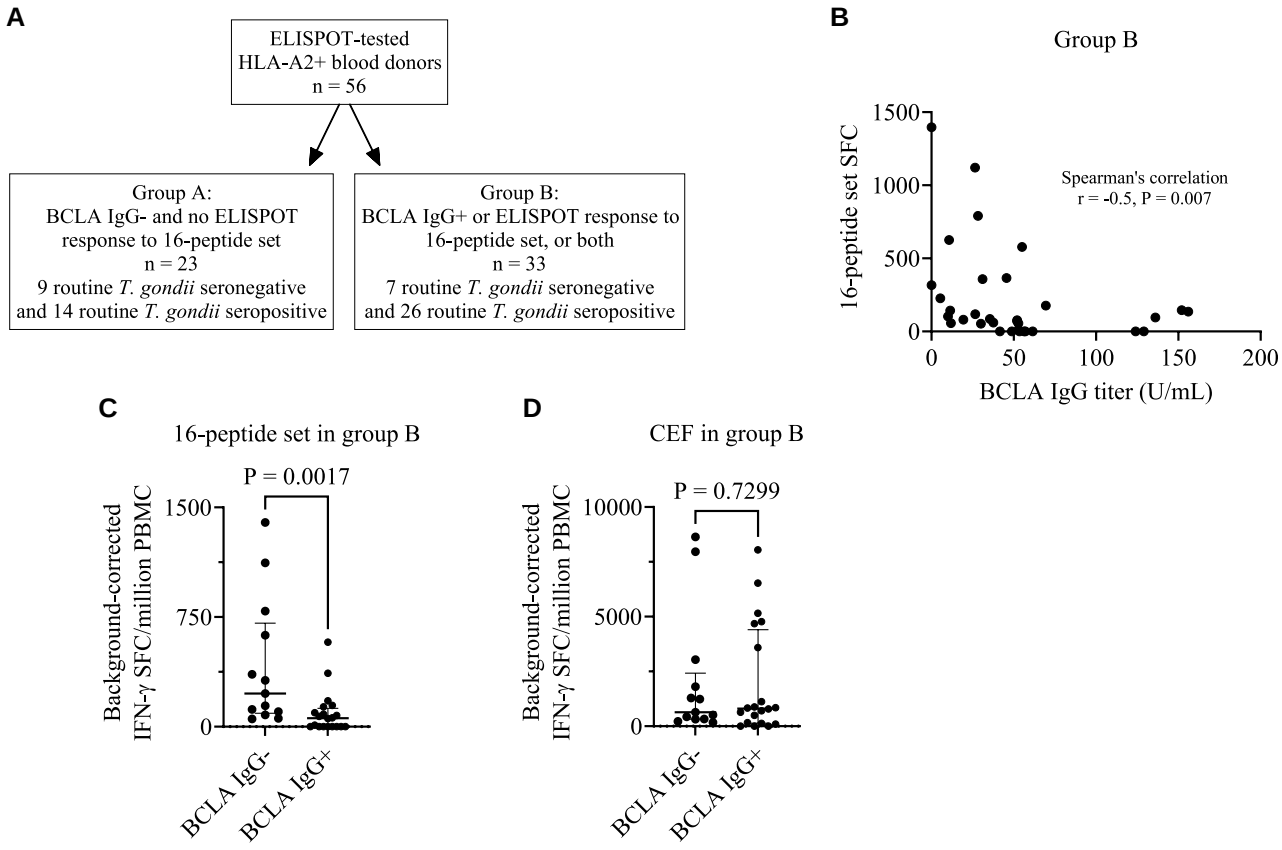

Figure 4. The 16-peptide set allows the monitoring of *T. gondii*–specific T-cell response during latency. *A*, Repartition of ELISPOT-tested HLA-A2+ subjects (n = 56). Group A (n = 23) comprised subjects who were negative for both BCLA serodiagnosis and ELISPOT response to the 16-peptide set, while subjects of group B (n = 33) were positive for BCLA serodiagnosis or ELISPOT response to the 16-peptide set, or both. *B*, Correlation between the sum of IFN-γ SFC/10^6 PBMC for the 16-peptide set and anti-BCLA IgG titers (IU/mL) in subjects of group B. A Spearman correlation was run to determine the relationship between these parameters; *r* and *P* values are displayed on the figure. *C*, Sum of IFN-γ SFC/10^6 PBMC for the 16-peptide set in anti-BCLA IgG-negative (n = 13) and anti-BCLA IgG-positive (n = 20) subjects of group B (n = 33). *D*, CEF-specific ELISPOT response in anti-BCLA IgG-negative (n = 13) and anti-BCLA IgG-positive (n = 20) subjects of group B.

binding affinity algorithms, or due to low intrinsic immunogenicity of HLA-A2-presented epitopes compared to other HLA-presented epitopes.

An intriguing observation of this work was that it revealed that 3 peptides (#14, #16, and #17) induced high ELISPOT responses in 2 out of 11 subjects who had non-detectable routine *T. gondii* and BCLA IgG titers. It is possible that antibody production in these individuals fell below detection while maintaining a long-lasting T-cell response, due to host's intrinsic factors and the persistence of T-cell activation by cysts, recrudescent tachyzoites or secondary infections. Supporting this hypothesis, previous *ex vivo* ELISPOT studies, using inactivated tachyzoites [36] or HLA-presented peptides [23] from *T. gondii*, reported T-cell responses in a few routine seronegative individuals. Another hypothesis could be that CD8+ T cells stimulated by these epitope peptides may have been raised against antigens derived from other microorganisms.

A noteworthy result of our study laid in the fact that the strongest CD8+ T-cell ELISPOT responses were detected in individuals who were negative for anti-BCLA IgG. In mice, BCLA-specific IgG titers were found to correlate with brain cyst burden [9]. Hence, one interpretation of our finding could be that protective CD8+ T-cell responses occur in some *T. gondii*–exposed individuals, thereby curtailing parasite load in the brain, and leading to absent or decreased seroreactivity toward bradyzoite antigens. In fact, cellular responses in the absence of seroreactivity have been described in the context of other chronic infection, such as hepatitis C virus (HCV) infection [37] and could be associated with viral containment in exposed seronegative subjects [38].

Our findings suggest potential applications of BCLA serodiagnosis and ELISPOT assays in screening and monitoring individuals with different clinical presentations of *T. gondii* infection. These assays could complement routine serological screening for latent *T. gondii* infection, especially in HIV-infected individuals and transplantation settings. They may also help assess reactivation risk that could be useful in ocular toxoplasmosis, which is a disease with substantial

worldwide prevalence and is linked to the immune response, particularly IFN-γ production [39, 40]. We hypothesize that monitoring T-cell responses and BCLA serostatus could inform the risk of ocular toxoplasmosis after congenital infection and the risk of potential recurrence following a first onset. To be able to generalize our findings across all HLA haplotypes, it would now be important to consider the development an HLA-independent ELISPOT assay. With a limited set of epitope peptide, a cost-effective way would be to prepare a lyophilized stock of a mixture of these peptides for ELISPOT assays. This type of strategy has already been developed for CMV-specific T-cell responses [41–43]. In conclusion, our work paves the way for the implementation of cellular immunity monitoring to *T. gondii* during chronicity, which will help decision-making by clinicians.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We thank Drs Florence Abravanel, Roland S. Liblau, and Hervé Pelloux for the critical reading of the manuscript. We thank F. L'Faqihi-Olive, V. Duplan-Eche, A.-L. Iscache, and H. Garnier from the flow cytometry core facility of Inserm UMR1291 for the technical assistance. Flow cytometry experiments were done at Inserm UMR1291 flow cytometry core facility connected to Toulouse Réseau Imagerie network, member of the France-BioImaging national infrastructure supported by the French National Research Agency (ANR-10-INBS-04).

Data availability. All data required for the conclusions are available in the article. Additional data and/or scripts are available from the corresponding authors upon reasonable request and appropriate agreements.

Financial support. This work was supported by institutional grants from Inserm, Agence Nationale de la Recherche (ANR-18-CE15-0015 MICCHROB; ANR-22-CE14-0053 NINTENDO; ANR-11-LABX0024 PIA PARAFRAP Consortium; ANR-22-CE15-0018-02 IMMUN'UP), Fondation pour la Recherche Médicale/Fondation Alzheimer (#ALZ201912009630), Fondation pour la Recherche sur le Cerveau AAP2021, and Agence Nationale pour la Recherche sur le SIDA et les Maladies Infectieuses Emergentes ANRS-MIE (ANRS0366). L. D., M. G. R., and M.-A. H. were supported by MSD Avenir (Project LatentToxoDiag, DS-2022-0017) and the Agence Nationale de la Recherche (Project Chairs of Excellence in Biology/Health, Project ToxoNeoSex, ANR-24-CHBS-0008).

Potential conflicts of interest. All authors: No reported conflicts.

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